Studies on the mechanism of F-promoted chromosome transfer in Escherichia coli K-12
by Louis Wallace Wendt

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Genetics
Montana State University

Abstract:
The consequences of two different (but not mutually exclusive) models of chromosome transfer by Hfr donors of E. coli have been examined experimentally. The finding that normal gene transfer can be carried out by HfrtS undergoing thymineless death in the presence of submaximal concentrations of thymine does not support the proposal that transfer depends on concurrent DNA synthesis. An attempt to stimulate the fertility of Hfr cultures by bringing all the Hfr to the (hypothetical) point at which transfer may be initiated by the use of chloramphenicol was unsuccessful. However, chloramphenicol treatment was found to reduce the ability of Hfr cells to form both stable and unstable pairs. These effects, and similar effects of phenethyl alcohol, have been traced to their action on donor-specific surface structures known as F pili. The inhibition of gene transfer by the male—specific bacteriophage MS-2, which is believed to attack the donor cell via its F-pili, supports the idea that these pili are the corridors, or vehicles, of transfer. finally, an examination of the available experimental evidence suggests that transfer may, in fact, be driven by processes associated with T-pili metabolism.

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ABSTRACT

The consequences of two different (but not mutually exclusive) models of chromosome transfer by Hfr donors of E. coli have been examined experimentally. The finding that normal gene transfer can be carried out by Hfr's undergoing thymineless death in the presence of submaximal concentrations of thymine does not support the proposal that transfer depends on concurrent DNA synthesis. An attempt to stimulate the fertility of Hfr cultures by bringing all the Hfr to the (hypothetical) point at which transfer may be initiated by the use of chloramphenicol was unsuccessful. However, chloramphenicol treatment was found to reduce the ability of Hfr cells to form both stable and unstable pairs. These effects, and similar effects of phenethyl alcohol, have been traced to their action on donor-specific surface structures known as F pili. The inhibition of gene transfer by the male-specific bacteriophage MS-2, which is believed to attack the donor cell via its F-pili, supports the idea that these pili are the corridors, or vehicles, of transfer. Finally, an examination of the available experimental evidence suggests that transfer may, in fact, be driven by processes associated with F-pili metabolism.

INTRODUCTION

The genetic basis of sexual differentiation in Escherichia coli is understood in some detail. Genetic exchange is unidirectional, proceeding from donors to recipients during conjugal pairing of the cells. The donor property is associated with the presence in the donor cell of genetic elements called promoters (Clark and Adelberg, 1962). Among these the most thoroughly studied is the sex factor, F, which may be cytoplasmic (F^+ types), have a chromosomal location (Hfr types), or alternate between these states (F'). If F is absent the cell is a recipient only (F^-) (Jacob and Wollman, 1961).

Genetic information is also available on the nature of F promoted chromosome transfer. The chromosome in all sex types appears to be circular. During conjugation involving, for example, an Hfr and F^-, the Hfr chromosome is thought to break at a point adjacent to F. The chromosome is then passed from the Hfr to the F^- lengthwise, starting with a particular end, the origin. Genes located at different distances from the origin are thus transferred to the F^- at different times, and in a sequence depending on their order along the chromosome. F itself is transferred last; that is, it is attached to what is now the 'tail' of the chromosome (Bouck and Adelberg, 1963). A basically identical sequence of events is thought to occur during F promoted chromosmal transfer by any donor type. "Conjugation tubes" through which the chromosome may be transferred have been observed connecting cells of opposite mating type under the electron microscope.

The physiological events necessary for transfer, however, are not clear. It is known that the genetic material in E. coli and the material transferred during conjugation are predominantly DNA (Jacob and Wollman, 1958; Garen and Skaar, 1958; Silver, 1963). Early work by Fisher (1957) was interpreted as showing that energy production by the donor cells only was required for transfer, and that the synthesis of neither DNA, RNA nor protein were involved. The first of Fisher's conclusions, which was based on experiments in which cells were aerated in buffer prior to mating to deplete their endogenous energy sources, has been criticized by Clark and Adelberg (1962). Since aeration in buffer may have other effects, they point out, this conclusion does not follow necessarily. Experiments by Fisher involving the use of dinitrophenol and anaerobic conditions, however, show energy production by mating pairs is necessary. The second of Fisher's conclusions is, in part, the subject of the present study.

Most attention in the past has been focused on the role of DNA synthesis (often equated with chromosome replication) in the transfer process, and two models of transfer have been proposed. Model 1, originating with A. J. Clark (Bouck and Adelberg, 1963) envisions that the break in the chromosome occurs at a particular time in its replication—at the end of what has been called the replication cycle. One of the new pair of chromosomes is then transferred to the F^- cell by an unspecified mechanism. Model 2, devised by Jacob and others (1963), proposes that transfer is driven by the replication process. The donor

chromosome (here assumed to be a DNA double-helix) is replicated from the origin by an enzymic apparatus associated with F. F and its associated "replication system" are fixed at a point inside the cell wall near the base of a conjugation tube so that the chromosome must move through this apparatus to be replicated. Early in conjugation the F system of replication is activated, and as replication begins one of the copies is diverted into the conjugation tube. Further replication pushes this copy through the tube into the recipient. The time at which the chromosome breaks is not clearly specified, but to be consistent with the model it would have to occur before, or as, the conjugal replication begins.

These models differ most clearly in the relationships they propose between DNA synthesis and the transfer process. On Model 1, DNA synthesis is required to complete chromosome replication prior to transfer, but may not be necessary during the transfer process itself. On Model 2, concurrent DNA synthesis is necessary for transfer. Model 1 also predicts a unique time in the cell division cycle during which chromosome transfer may be initiated—the period between the end of one DNA replication and the start of another. This feature may be accommodated to Model 2, but is not required by it.

The results of attempts to test these models using treatments which inhibit DNA synthesis are apparently contradictory. Bouck and Adelberg (1963) performed an experiment which is consistent with the existence of a particular time in the DNA replication cycle during

which transfer is initiated. Jacob and co-workers (1963) offer evidence that it is not. The experiments of Roeser and Konetzka (1964) were interpreted as consistent with both models, but their interpretation seems to be inconsistent with certain aspects of their own results. Reassuringly, all of these interpretations are open to objections. For example, phenethyl alcohol (PEA) has been used to inhibit DNA synthesis (Bouck and Adelberg, Jacob et al., Roeser and Konetzka), but it affects RNA and, indirectly, protein synthesis as well (Nonoyama and Ikeda, 1964; Treick and Konetzka, 1964; Rosenkranz and others, 1965). Furthermore, as already indicated, these models are not mutually exclusive. It could be that DNA synthesis is necessary for transfer, but that such synthesis must be initiated at a particular time in the cell division, or chromosome replication, cycle.

Our own experiments were aimed at testing the dependence of transfer on concurrent DNA synthesis by depriving Hfr cells of thymine during conjugation, and the existence of a unique time of initiation of transfer by the use of treatments which might synchronize cell division or DNA replication. These have led, unexpectedly, to the recognition of the importance of donor-specific surface structures known as F-pili (Brinton et al., 1964) in conjugation, and to further experiments aimed at understanding F-pili function.

MATERIALS AND METHODS

Bacterial and bacteriophage strains

The bacterial strains used were derivatives of Escherichia coli K-12. MSC 603 is a thymineless derivative of CS 101, which is itself a derivative of the Cavalli Hfr strain (Hfr C; Cavalli, 1950). MSC 603 thus requires thymine and methionine, ferments lactose, and is streptomycin sensitive. W1177 is a multiply-marked K12 derivative via Y53 (Lederberg, 1947). It requires threonine, leucine and thiamine, does not ferment lactose and is streptomycin resistant.

MS-2, a donor specific bacteriophage, was donated by Dr. A. J. Clark.

Media and supplements

Final concentrations are given as grams/liter of distilled water unless otherwise indicated. Media were sterilized in an autoclave at 18 pounds pressure for 20-25 minutes.

Nutrient Agar: Difco Nutrient Broth 8, NaCl 5, agar 15.

Pennassay Broth (PB): Bacto-Beef Extract 1-5, Bacto-Yeast Extract 1.5, Bacto-Peptone 5.0, Bacto-Dextrose 1.0, NaCl 3.5, K_2HPO_4 3.6, KH_2PO_4 1.3.

Eosin Methylene Blue Agar (EMB): Bacto-Casitone 8.0, Yeast Extract 1.0, NaCl 5.0, K_2HPO_4 2.0, Eosin Y 0.4, Methylene blue 0.065, sugar 10.0, agar 15.0.

Davis Minimal Agar (DMA): K_2HPO_4 7.0, KH_2PO_4 2.0, crystalline sodium citrate 0.5, crystalline $MgSO_4$ 0.1, crystalline $(NH_4)_2SO_4$ 1.0,

sugar 1.0, agar 15.0. The salts were added to 500 ml of water and sterilized. If the sugar added was glucose, it and the agar were added together to 500 ml of water and mixed with the salt solution after sterilization. If the sugar added was lactose, the agar was added to 490 ml of water alone and sterilized, while one gram of lactose was dissolved in 10 ml of sterile water and added to the agar after sterilization.

Saline: NaCl 8.6.

Streptomycin (Dihydro-streptomycin sulfate): 250 mg/ml.

Aminopterin (AM): As part of the procedure for isolating thymine-less mutants AM was added to certain cultures to give a final concentration of 0.2 mg/ml.

Tris-glucose Minimal Medium (TG): NaCl 5.4, KCL 3.0, NH_4Cl 1.1, $CaCl_2$ 0.011, $MgCl_2$ 0.095, $FeCl_3$ 0.00162, KH_2PO_4 0.0872, Na_2SO_4 0.0227, Tris (hydroxymethyl) aminomethane 12.1. The pH was adjusted to 7.2 with concentrated HCl and the solution sterilized (T). Just before using, 9 parts were mixed with 1 part sterile glucose solution containing 20.0 grams/liter glucose.

TG*: TG supplemented with thymine, methionine, threonine, leucine and thiamine.

Growth factors: The following were added to DMA or TG when required: methionine 0.02, thymine or thymidine 0.02, thrionine 0.04, leucine 0.02, thiamine 0.0001.

Inhibitors: To inhibit conjugation the following were added to

liquid media to give the final concentrations indicated: chloramphenical (CAP) 0.05 mg/ml, phenethyl alcohol (PEA) 0.3%.

Storage of bacterial and bacteriophage strains and preparation of working cultures and lysates

Bacterial strains. Bacterial strains were stored on nutrient agar slants at 4° C and transferred every few months. The transfer always included single colony isolation and tests for the relevant growth requirements.

In some cases—when a particular strain was to be used daily, or to be able to observe individual colony morphology of thymineless mutants—strains were streaked from these slants onto nutrient agar plates and liquid cultures inoculated by picking patches of dense growth or many colonies. All other inoculations were made directly from the stock slants.

Unless stated otherwise cultures were incubated at 37° C. Pennassay broth cultures were grown overnight (12-16 hrs.). Log phase cultures in this medium were obtained by diluting 1/10-1/20 from an overnight culture and incubating 90-120 minutes. Overnight TC cultures were started from a heavier inoculum and incubated longer (16-20 hrs.), the exact time depending on the particular batch of medium. Log phase TG cultures were also started by 1/10-1/20 dilution from an overnight culture, but needed to be incubated 3-5 hours, depending on the particular experiment or the batch of medium. For use in conjugation experiments in TC* medium, F^- cultures were routinely grown to late log

or early stationary phase to maximize the yield of viable cells. This procedure has no apparent effect on F^- fertility.

Bacteriophage techniques. Phage lysates were obtained by the method described by Adams (1959). After collection, the phage suspensions were centrifuged to remove cells, and the supernate decanted and treated with chloroform. The supernate was again poured off and incubated in an open petri dish, or aerated for a short time, to remove the remaining chloroform. The final lysate was stored at 4° C.

Lysates were assayed by the agar-layer method at appropriate dilutions on nutrient agar.

Phage adsorption was measured by adding phage to an excess of cells in a growing culture and following the decrease in plaque forming units as detailed by Adams: the culture was diluted 100 fold into cold medium to stop adsorption. These dilutions were then centrifuged to sediment the cells, and then assayed by plating 0.1 ml of the supernate with 0.1 ml of an overnight culture of MSC 603 using the top layer method.

Isolation of thymineless mutants

Thymineless mutants were isolated by the general method of Okada et al. (1962). Dense cultures in TG medium and thymidine were prepared by aeration for 16 hours after inoculation from an overnight culture. These were then diluted 1/500 into the same medium lacking thymine and containing 0.2 mg/ml aminopterin and aerated for 24 hours. If, at the end of this time, the cultures were turbid, they were

discarded. If, however, there was no visible growth, 0.5 ml of thymine (2 mg/ml) was added to 5 ml of the culture and incubation continued for 48 to 72 hours more. These final cultures were streaked, or diluted and plated, on nutrient agar and incubated until colonies appeared.

Thymineless colonies of MSC 603 could be recognized immediately by a characteristic 'thin', or translucent, appearance on nutrient agar (Okada et al., 1962), and were tested for their thymine requirement by picking and streaking, or spotting, on minimal medium complete for this strain (i.e., lacking only thymine). The thin colony phenotype was lost in the course of repeated transfers, but no attempt to study this phenomenon has been made.

Mating procedures

Matings to determine the fertility of MSC 603 under different conditions and to follow the kinetics of transfer of certain markers were performed by the method developed by de Haan and Gross (1962). Log-phase Hfr cultures and F^- cultures were mixed to give concentrations of 1-2 x 10^7 Hfr/2-4 x 10^8 F^- per ml; when using Pennassay broth cultures the Hfr was simply diluted 1/10 into an aliquot of the F^- culture. When using TG* medium, aliquots of the F^- were centrifuged and the pellets resuspended with an appropriate volume of Hfr cells at 2 x 10^7/ml to initiate the mating. This procedure was followed to avoid using Hfr cultures which had gone out of log phase.

After mixing, the cultures were incubated five minutes to allow the cells to pair and then diluted to stop further pairing. Cultures

mated in TG* medium were diluted 100 fold into the same medium. Cultures mated in Pennassay broth were diluted into 10^{-2} PB (PB diluted 1/100 with saline). Depending on the experiment, samples were removed from the diluted cultures either at five minute intervals, or at 30 or 60 minutes to assay recombinants, and, in some cases, viable cells.

Recombinants were assayed by spreading 0.1 ml samples of the diluted culture on appropriately supplemented DMA. Where lac$^+$ transfer was measured the plates lacked glucose, and were supplemented with lactose, threonine, leucine and thiamine. Where leu$^+$ transfer was measured the plates contained glucose and were supplemented with leucine and thiamine. In all cases streptomycin was added to kill the donor cells and prevent further transfer.

Samples removed from mating cultures are usually blended in a high speed mixer before plating. This treatment is thought to be necessary to separate mating pairs and prevent transfer from being completed on the plates by those pairs which have not completed it at the time of sampling. In our own experiments blending has not been used, since it was found to be possible to obtain identical kinetics with or without it. The only difference between the data obtained in the two situations is that without blending, a few 'background' colonies may be seen on plates made at times before the marker being selected enters. It appears that the spreading of the samples and the presence of streptomycin on the plates combine to prevent transfer in greater than 90% of the pairs plated at such times.

RESULTS

The effects of thymine deprivation upon conjugation

Effects on 60 minute recombinant yield. In the absence of thymine the amount of DNA made by thymine-requiring strains of E. coli is small (Barner and Cohen, 1956; Korn and Weisbach, 1962; Seno and Melechen, 1964). In addition there is, typically, a 30-60 minute lag before the cells start to die, whereas DNA synthesis proceeds at its new and lower rate from time zero. If transfer depends on concurrent DNA synthesis it might then be predicted that the fertility (the number of cells able to transfer a given gene measured under some standard set of conditions) of an Hfr culture undergoing thymine deprivation would decay faster than cell viability. This assumes DNA synthesis in the donor cells is not restored in some way (e.g., by feeding) upon mixing with the F^- cells.

To test this prediction, experiments designed to measure the decay of fertility of MSC 603 in the absence of thymine were performed as follows: A log-phase culture of MSC 603 in TG* was washed twice by centrifugation, resuspended in the same medium lacking only thymine, and incubated at 37° C. Samples were removed at 30 minute intervals, diluted in saline, and spread on nutrient agar to measure viable cells. At the same times samples were removed and mated with W1177 grown in the same medium to determine relative fertility. Mating cultures in TG* lacking thymine were prepared as described in Materials and Methods. Samples (0.1 ml) were removed after one hour and plated directly on

appropriately supplemented DMA to score *lac*$^+$ recombinants. Thus, the criterion of fertility in these experiments is the number of *lac*$^+$ recombinants formed after 60 minutes of mating. By this time the number of these recombinants has reached its maximum value, as will be seen in subsequent experiments.

The results of a typical experiment are given in Figure 1. Two features of the results are immediately apparent: (1) Unexpectedly, fertility seems to be enhanced initially. (2) The rate of fertility decay appears to be less than the rate of thymineless death.

The first feature has been found to be an artifact of the washing procedure. The effect of this procedure in reducing fertility and the recovery of fertility after the final resuspension is shown in Table Ia. The experiment was performed as described above except that an additional sample was removed just before washing, and only two samples were taken after resuspension--one immediately and the other after one hour. It can be seen that the fertility was reduced to one-third of its original value by washing, but returned to this value after one hour of incubation. The results of a second experiment recorded in Table Ib show that the fall in fertility is due only to centrifugation and resuspension and not some other aspect of the washing procedure. A log-phase culture of MSC 603 in TG* was centrifuged and resuspended twice in the same medium. The centrifugation was performed in an incubator room with all materials at 36-37° C. Samples were removed to measure fertility and assay viable cells before and after each

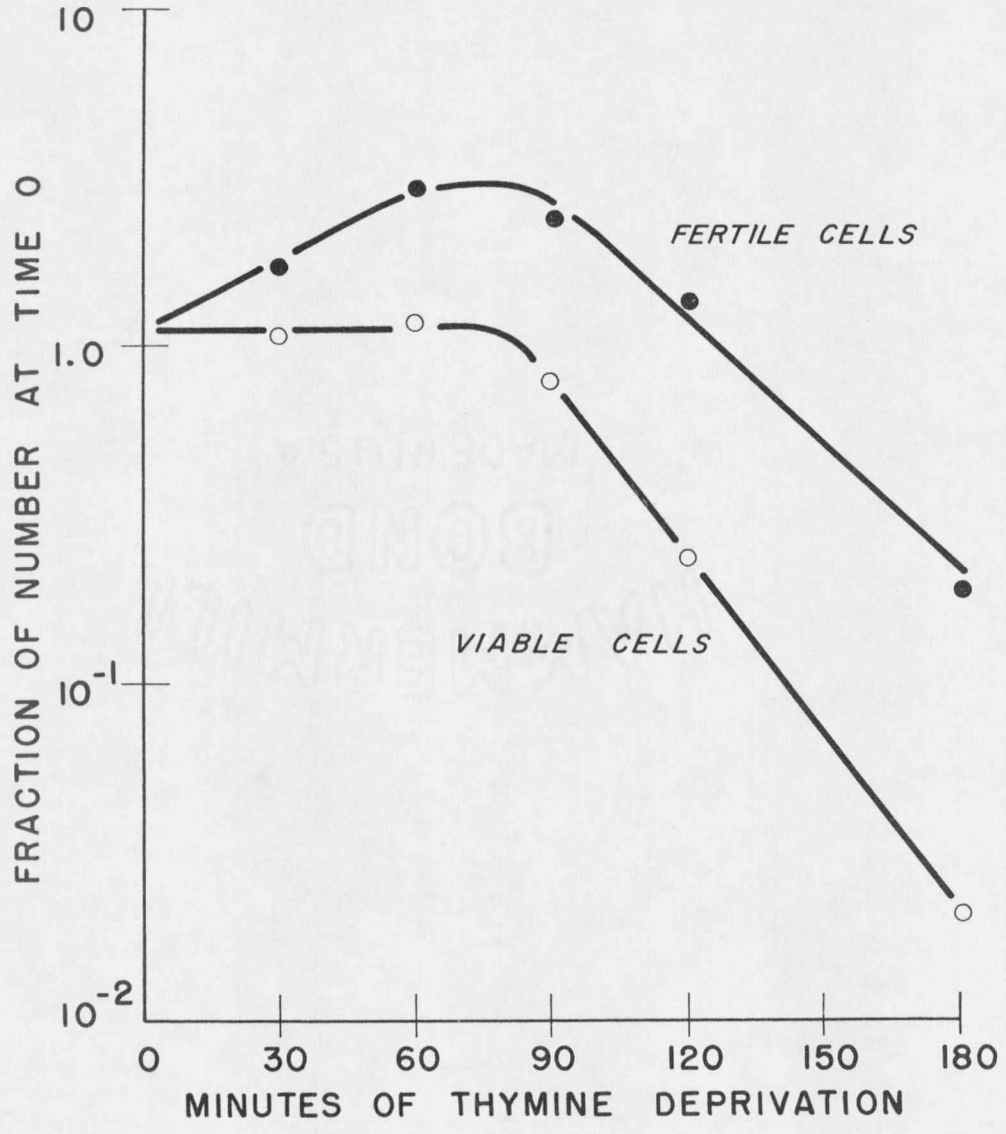

Figure 1. Decay of viability and fertility of a donor strain during thymine deprivation. Log phase MSC 603 were deprived of thymine at time 0. At intervals, samples were removed to measure viable cells and fertility. "Fertile cells" were those which yielded lac$^+$ recombinants when mated for 60 minutes with W1177. All points are averages of two independent determinations.

Table I. Effect of washing on Hfr fertility.

Time of sample	Viable cells x 10^{-5} (average of 2 independent determinations)	Recombinants x 10^{-3} (average of 3 independent determinations)	Relative Fertility (Recombinants/ Viable cells x 10^2)
a.			
Before washing	206	310	1.69
Immediately after resuspension	156	79	.51
One hour after resuspension	148	275	1.86
b.			
Before centrifugation	172	383	2.22
After first centrifugation	175	129	.73
After second centrifugation	180	107	.59

MSC 603 was grown to log phase in TG*, and a sample removed to assay viable cells, and to mate with W1177. In a. the culture was then washed by centrifugation and resuspended in medium without thymine. In b. resuspension was in medium with thymine. Samples were also removed for viable cell assay and for mating at the later times indicated.

centrifugation. After the first centrifugation the fertility dropped to about one-third of its original value. Only a slight additional fall occurred after the second centrifugation. This effect can be attributed to mechanical breakage and loss of a portion of the donor cells F-pili when the cells are packed together and subsequently re-suspended. A correlation between the loss of these structures and the loss of fertility has been reported by Brinton and others (1964) and was also observed indirectly in the chloramphenicol experiments to be described later. Further information on F-pili, and experiments implicating them in gene transfer are reported in later sections of these results.

Since 60 minutes appears to be sufficient to undo the effects of centrifugation, and since thymineless death does not begin until after 60 minutes, a comparison of the kinetics of decay of fertility and viability after this time should establish their relative rates. The results of three experiments are included in Figure 2. Because of variability in fertility from experiment to experiment, the ratio between fertile and viable cells has been plotted. From this comparison it is clear that the decay in ability to form lac^+ recombinants after 60 minutes is slower than the decay of viability, just the opposite of our prediction. This finding, in fact, suggests that cells not capable of giving rise to colonies on nutrient agar may still be competent to transfer genes.

Thymineless death in mating mixtures. The results given in

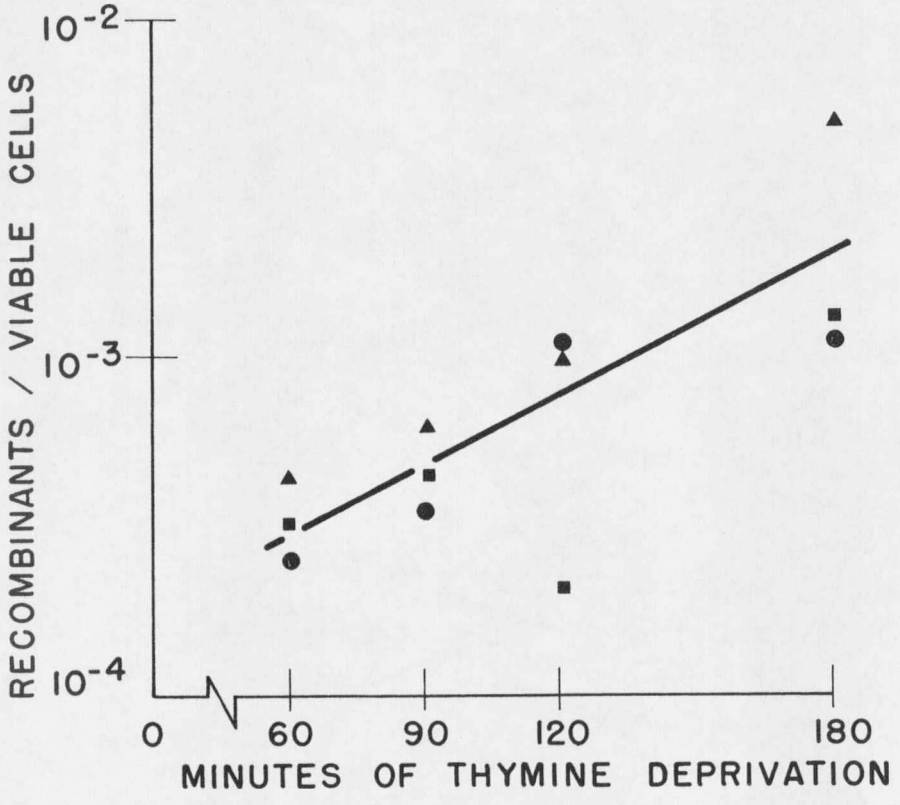

Figure 2. The relationship between viability and fertility after varying periods of thymine deprivation. Three experiments like that illustrated in Figure 1 are involved. Each is represented by a different symbol.

the preceding section are not those predicted on the basis of Model 2. However, an important assumption remains to be examined: that DNA synthesis in the donor is not restored upon mixing with the F^- cells. DNA synthesis has not been measured directly; instead the rate of thymineless death has been taken as a measure of its inhibition. Thus, we have compared the rate of thymineless death of MSC 603 in mating mixtures with that of MSC 603 alone.

A log phase culture of MSC 603 in TG* was washed once by centrifugation and resuspended in T buffer. Three aliquots of this suspension were mixed with three pellets prepared from a W1177 culture grown in the same medium to give three mating mixtures. These mixtures and three equivalent volumes of MSC 603 alone were incubated at 37° for five minutes and then diluted 100-fold into TG* lacking thymine. The death of MSC 603 in these six cultures was followed by removing samples at one hour intervals, diluting appropriately, and plating on EMB-lactose to score the viable $\underline{lac}^+$ (MSC 603) cells.

As can be seen from Figure 3, the rate of thymineless death of MSC 603 mixed with W1177 is markedly slower than the rate of death of MSC 603 alone, suggesting that mixing with W1177 under these conditions does allow appreciably more DNA synthesis in the donor cells than would otherwise occur. This effect may be due to thymine associated with the F^- pellet, since the amount of thymine routinely added to cultures (20 mg/ml) is in excess of that necessary for maximal growth. The concentration of contaminating thymine must, however, be

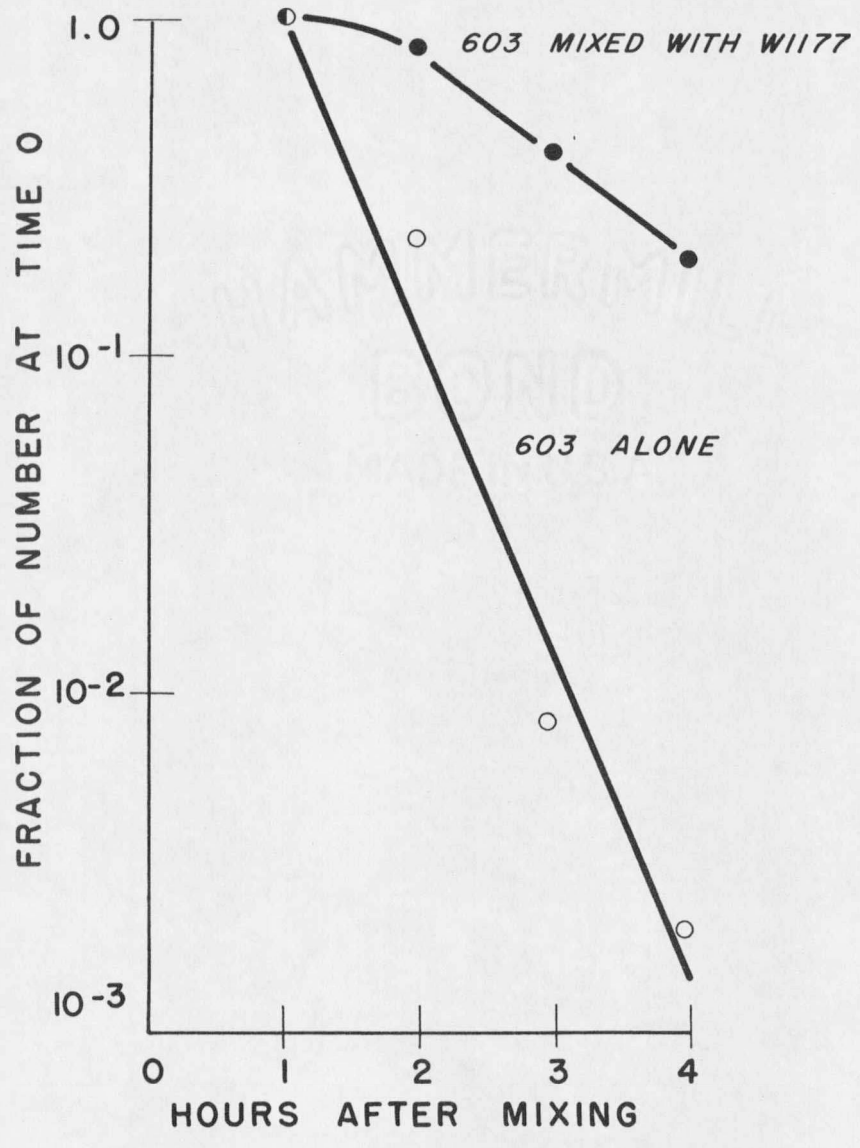

Figure 3. The thymineless death of MSC 603 in mating mixtures. A log phase culture of MSC 603 was prepared for thymine deprivation and mated with W1177 as described in the text. Samples were removed to assay viable MSC 603 cells at one hour intervals. All points are averages of three independent cultures.

below the level required to sustain growth, since some thymineless death is observed.

Kinetics of transfer during thymine deprivation. If transfer is dependent on DNA synthesis, this submaximal thymine concentration should lead to slower transfer. To test this a log phase culture of MSC 603 in TG* medium was prepared for thymine deprivation as described previously. Samples were removed immediately after the final resuspension (start of thymine deprivation) and after one hour of deprivation, and mated with W1177 in TG* lacking thymine. Following the routine dilution at five minutes to prevent further pairing the mating mixtures were sampled at five minute intervals, and 0.1 ml plated directly on the appropriate media to score lac^+ and leu^+ recombinants.

As seen in Figure 4, the times of entry and maximum transfer of lac^+ and leu^+ by Hfr's starved of thymine for one hour, and by those which had not been starved are identical. (The greater fertility of the former has been discussed in the preceding section.) Thus, if transfer is dependent on DNA synthesis, the latter must resume immediately and at close to its normal rate even when the cells are provided with submaximal levels of thymine after one hour of deprivation. Since thymineless death does occur, however, this appears unlikely, and hence suggests that Model 2 needs further substantiation.

Effects of protein synthesis inhibitors upon conjugation

Effects of chloramphenicol. Protein synthesis in E. coli may be involved in both the formation of mating pairs and in the transfer

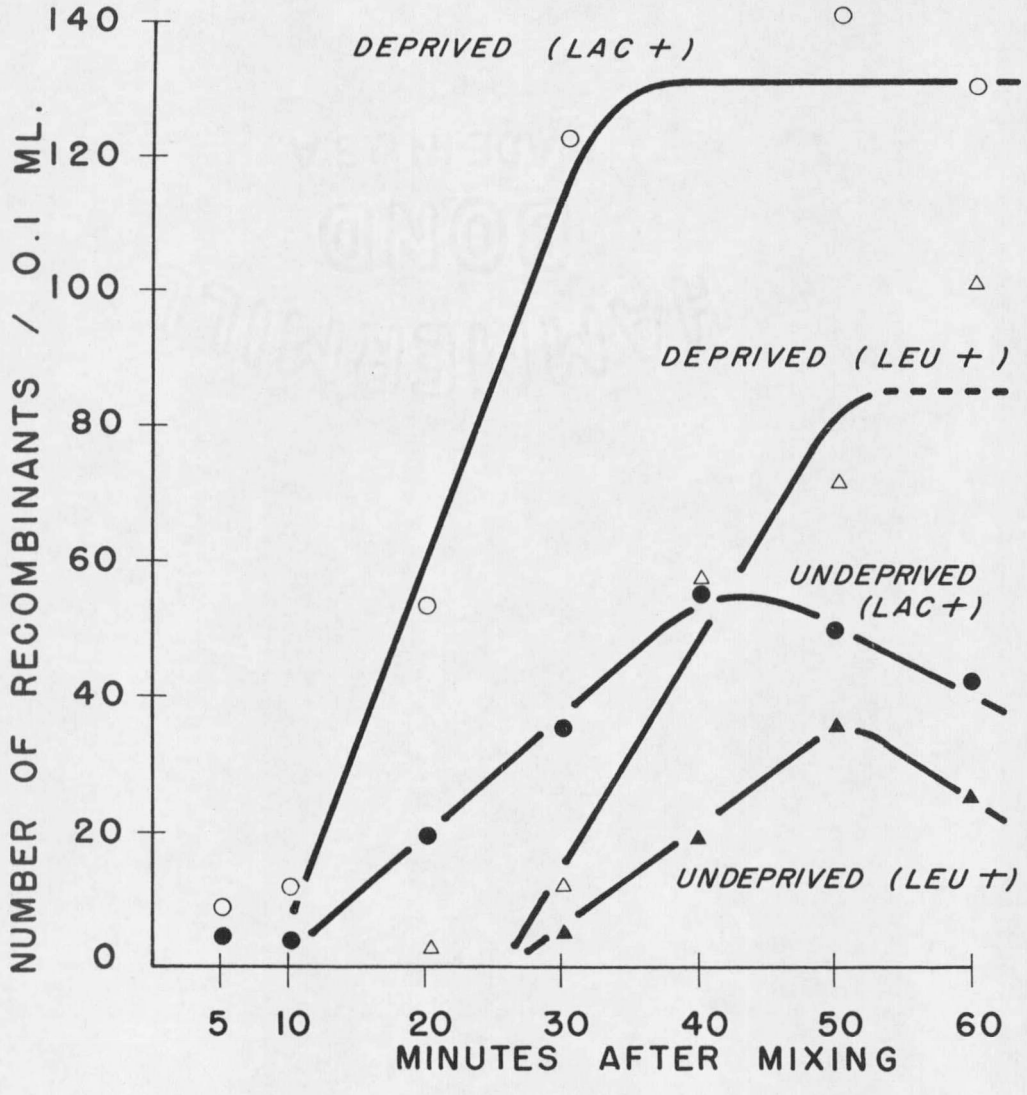

Figure 4. Kinetics of transfer of <u>lac</u>$^+$ and <u>leu</u>$^+$ by MSC 603 with and without prior thymine deprivation. A log phase TG* culture of MSC 603 was deprived of thymine as described in the text and samples removed for mating with W1177 at 0 and after 60 minutes of deprivation. <u>Lac</u>$^+$ and <u>leu</u>$^+$ transfer were then followed in both mating cultures. All points are averages of two independent determinations.

of the chromosome. Brinton et al. (1964) reported that donor cells of E. coli produce specific surface structures called F-pili. These pili appear to resemble other E. coli pili which are protein rods with an axial hole of approximately 25 Å diameter. They have been found necessary for conjugation and may function merely as organelles of pairing. As Brinton and his co-workers have proposed, they may also be directly involved in the transfer process by (1) serving as the conjugation tube through which the chromosome is passed from donor to recipient, or (2) growing out from the donor cell and into the recipient carrying the chromosome with them.

In addition, Maaloe and Hanawalt (1961) suggested that a period of protein synthesis is required between successive rounds of DNA replication in unmated bacteria. This suggestion was strengthened by the finding that chloramphenicol (CAP) prevents the initiation of new cycles of DNA replication, which begin at a particular point on the E. coli chromosome (Lark and Lark, 1964). Nagata (1963) has presented evidence which indicates that the site of initiation of replication and the point of origin in an Hfr chromosome may be identical, or at least closely associated.

In view of the preceding information, inhibition of protein synthesis in an Hfr culture might affect the conjugation process in either of two general ways. It might affect the ability of Hfr cells to form pairs or to transfer by inhibiting F-pili synthesis; or, it might affect transfer less directly, by interfering with the opening

or reclosing of the chromosome occurring between DNA replications proposed by Model 1. The second possibility leads to an interesting prediction. By blocking the initiation of new replication cycles, CAP might arrest most of the cells in a culture at this point, thereby effectively synchronizing them with respect to DNA replication. If this synchrony were maintained after removal of CAP, matings involving Hfr cells pretreated with the inhibitor might exhibit a burst of transfer exceeding that observed in control matings. This burst might occur either at the usual time of entry of the marker under consideration, or after a delay corresponding to the sum of the replication interval and the normal time of entry, depending on whether opening of the chromosome occurs after or before the block imposed by CAP.

Accordingly, the effects of different periods of CAP treatment on subsequent transfer by MSC 603 were examined. CAP was added to log-phase cultures of MSC 603 in TG* and samples removed at different times for mating with W1177 grown in the same medium. Because of the method of mating (resuspension of a W1177 pellet with a small volume of Hfr culture) CAP was present during the five minutes allowed for pair formation in these matings of MSC 603 which had been pretreated with the inhibitor. The routine 100-fold dilution of the mating cells after five minutes serves to remove the inhibitor.

In Figure 5 are presented the effects of pretreating MSC 603 with CAP for 0, 60, and 90 minutes upon the transfer of the $\underline{lac}^+$ gene. The experiment shown in Figure 5a reveals that pair formation in the

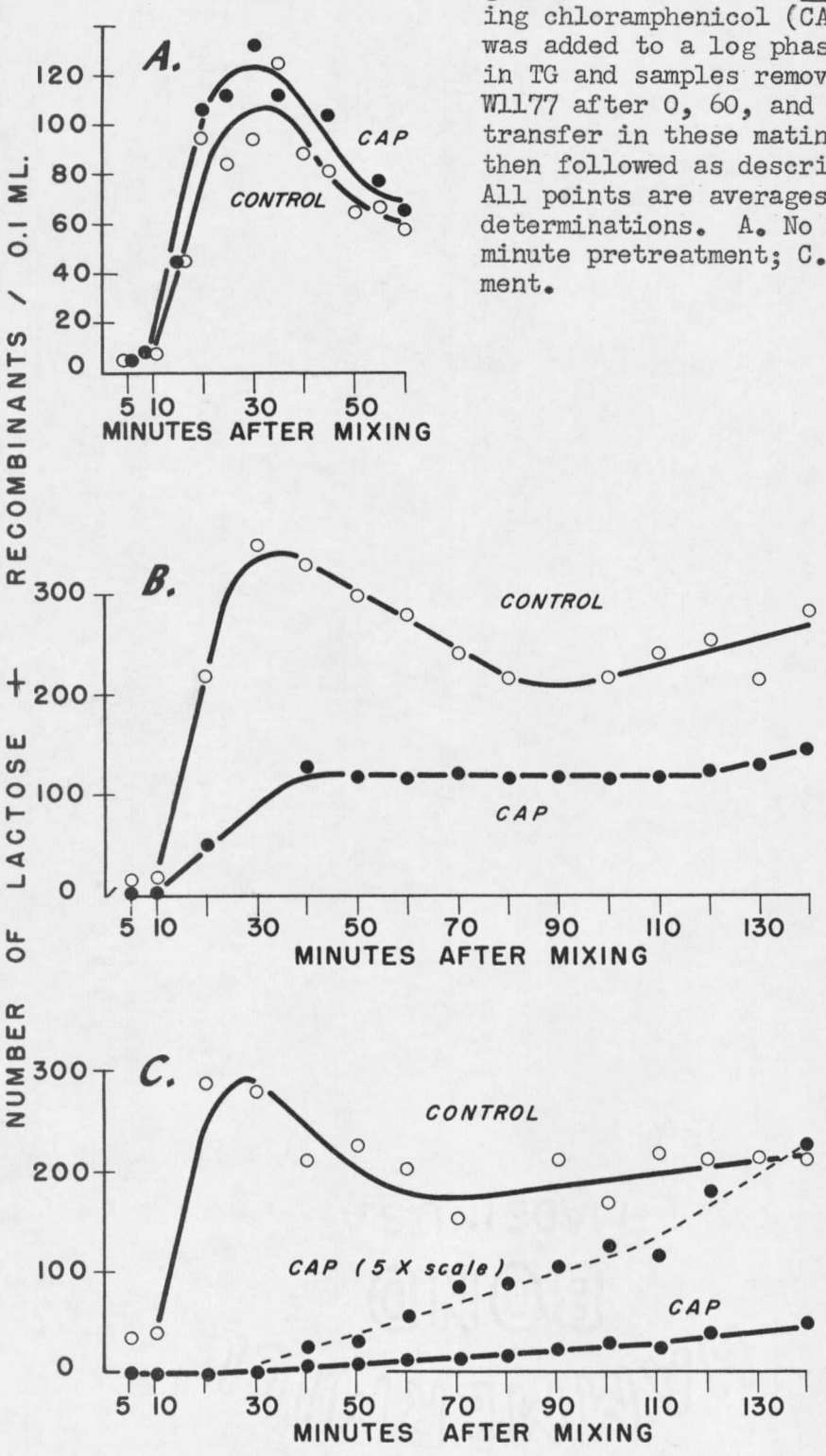

Figure 5. Kinetics of Lac$^+$ transfer following chloramphenicol (CAP) treatment. CAP was added to a log phase culture of MSC 603 in TG and samples removed for mating with W1177 after 0, 60, and 90 minutes. Lac$^+$ transfer in these mating cultures was then followed as described in the text. All points are averages of two independent determinations. A. No pretreatment; B. 60 minute pretreatment; C. 90 minute pretreatment.

presence of CAP by MSC 603 not previously treated with the inhibitor is, if anything, slightly increased. The fall in the number of recombinants between 30 and 60 minutes in this experiment is of the type observed by deHaan and Gross (1962). It was interpreted by them as due to the separation (uniquely in liquid minimal media) of some of the mating pairs, resulting in the withdrawal from the F^- cell of the chromosome segment containing the marker under consideration. In the following discussion, their interpretation of this phenomenon is accepted, and such pairs are referred to as unstable pairs.

In Figure 5b the effects of a 60 minute pretreatment are shown. The control mating is equivalent to the experimental mating in Figure 5a and exhibits a similar drop in the number of recombinants between 30 and 60 minutes. In the mating involving the pretreated MSC 603, however, this fall does not occur. A possible interpretation of this result is that 60 minutes in CAP prevents the Hfr cells from forming unstable pairs. That this treatment may also prevent the formation of what may be called stable pairs is suggested by the lower number of recombinants formed at later times by the treated cells. Finally, there is no indication of a delayed transfer by cells in the treated culture. Although the number of recombinant colonies increases after 120 minutes, it does so in both cultures, and the increase appears to begin earlier in the control. This increase, therefore, is most likely due to the multiplication of existing recombinants, rather than to late transfer.

Delayed transfer may, however, occur in matings made with Hfr cells pretreated for 90 minutes with CAP (Figure 5c). In this mating, the ability to form pairs appears to have been so reduced that the detection of normal $\underline{lac}^+$ transfer is not possible. However, a delayed increase in the number of recombinant colonies was observed. This increase is not of the type which might be predicted on Model 1. Nonetheless, it has been consistently observed in three experiments and deserves further comment.

It appears from the kinetics of this increase that it could not be due primarily to the multiplication of existing recombinants: The increases in the control cultures, which are slower and appear to begin later, probably <u>are</u> due to recombinant multiplication. Explaining the former increase in this way would lead to the conclusion that prolonged treatment of Hfr's with CAP stimulates the multiplication of recombinants formed by them. But since a similar increase is not observed after the 60 minute treatment this is unlikely. It is also unlikely that this increase could be due to delayed pair formation, since the cell concentration in these cultures was below the lowest at which pair formation has been detected (Hayes, 1957). We conclude, therefore, that it is due to transfer, but transfer of an unusual kind. There is no initial rise period, followed by a level period representing completed transfer by all pairs formed. Rather, transfer by some pairs is still being completed 100 minutes after that in other pairs. Most probably, this abnormal transfer is characteristic only of Hfr cells that have been treated for an extended period with CAP. On the other

hand, it is possible that such transfer occurs in all matings and is observable only if normal transfer is reduced to the limits of detectability.

In summary, these experiments reveal three ways in which CAP may affect conjugation: (1) By inhibiting unstable pair formation and (2) by inhibiting stable pair formation (both of which are evident after 60 minutes pretreatment), and (3) by slowing the transfer process (observable only after 90 minutes pretreatment). The first two effects could reflect the destruction and/or non-replacement of F-pili. However, the rates of decay of the two processes are markedly different; unstable pair formation is apparently eliminated by 60 minutes pretreatment, whereas the delayed transfer observed after 90 minutes pretreatment indicates that stable pair formation has not been entirely eliminated by this time. Since no burst of transfer was seen following any treatment, even at times long after mating, no support for Model 1 is provided. The abnormal transfer observed after 90 minutes pretreatment probably reflects a complicated poisoning of the cell machinery from which recovery is very slow. If so, the system is simply not adequate to test Model 1.

Effects of phenethyl alcohol. Treick and Konetzka (1964) have found that phenethyl alcohol (PEA) inhibits DNA synthesis in a manner similar to chloramphenicol (CAP). Current cycles of DNA synthesis may be completed in the presence of PEA, but no new cycles may be initiated. Thus, it is likely that PEA, also, stops the synthesis of DNA by

inhibiting protein synthesis. Effects of PEA on protein, and on RNA, synthesis have been reported (Treick and Konetzka, 1964; Nonoyama and Ikeda, 1964; Rosenkranz et al., 1965).

Roeser and Konetzka (1964) have studied transfer following prolonged PEA treatment and their main findings will be treated in the Discussion. The present experiments have revealed a very rapid effect of PEA not reported by these workers.

In the same experiment shown in Figure 5a, PEA was added to an aliquot of the MSC 603 culture, and a sample removed immediately for mating with W1177. As in the other matings, this mating mixture was diluted 100-fold after 5 minutes to prevent further pair formation, and to effectively remove the inhibitor. The transfer curve obtained after even this short exposure of the Hfr cells to PEA is strikingly different from that found for the control, and resembles (in fact) the transfer curve observed after the 60 minute CAP treatment. Thus, within the 5 minute period in the presence of PEA, the ability of the Hfr cells to form unstable pairs appears to be destroyed. The speed of this effect suggests that it may be brought about through some action on the cell surface. However, PEA appears to inhibit messenger RNA synthesis (Rosenkranz et al., 1964), and the inhibition of the synthesis of this, or some other unstable internal substance, might also be involved here.

Effects of protein synthesis inhibitors upon MS-2 phage adsorption

The most dramatic effects of pretreatment of donor cells with

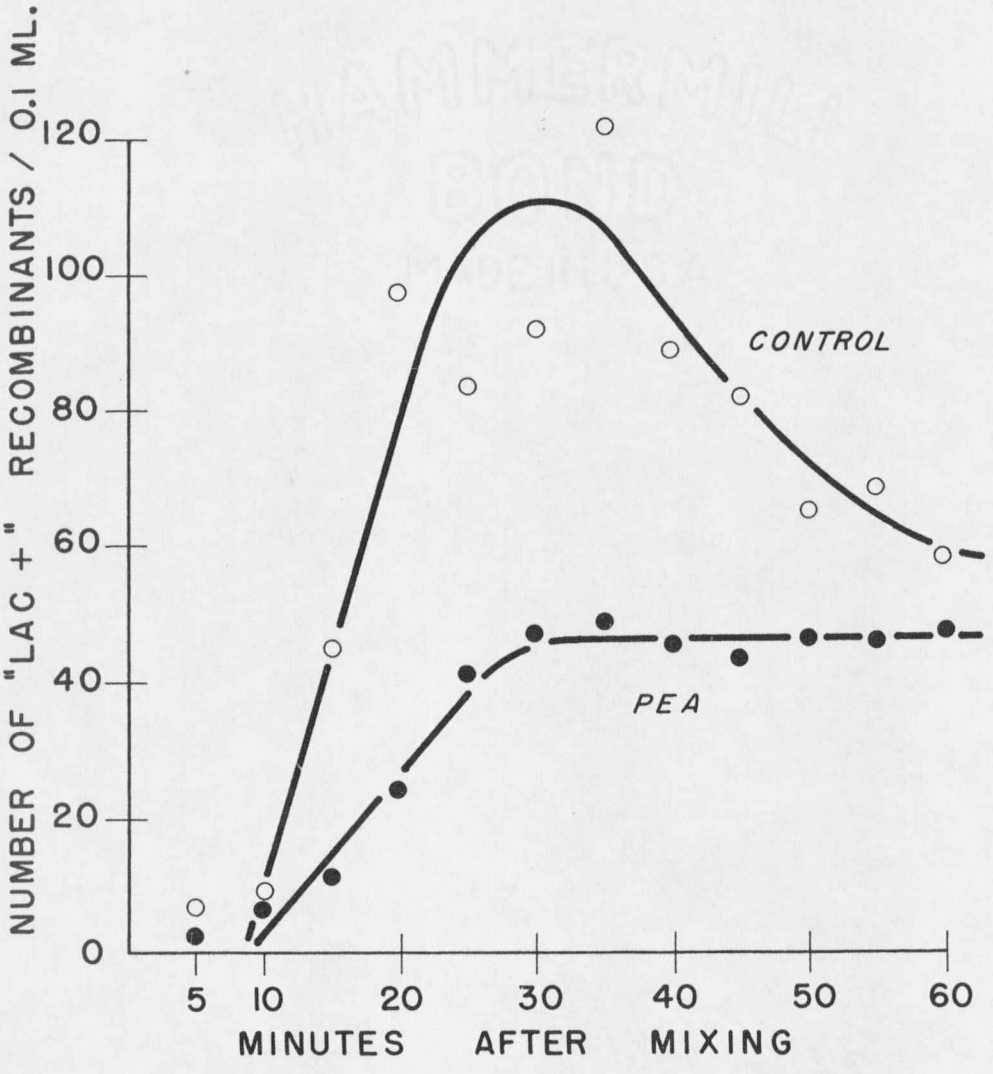

Figure 6. The effect of phenethyl alcohol (PEA) on pair formation. PEA was added to an aliquot of an MSC 603 culture in TG* and a sample removed immediately for mating with W1177. The kinetics of <u>lac</u>$^+$ transfer were then followed. The control curve is the same as that in Figure 5a. All points are averages of two determinations.

chloramphenicol (CAP) appear to be (1) the elimination of their ability to form unstable pairs, and (2) the depression of their ability to form stable pairs. Phenethyl alcohol (PEA) also appears to eliminate the ability of donor cells to form unstable pairs, and to do so much more quickly than CAP. In order to test these inferences further, and to gain further insight into the pairing process, the effects of these two inhibitors upon the ability of donor cells to adsorb phage MS-2 were studied.

MS-2 is one of those phages which specifically attack donor cells (Loeb and Zinder, 1961). Brinton et al. (1964) have found that these phages adsorb to the cells' F-pili. Since, as has been noted previously, these pili contain protein, a reasonable explanation of the effects upon pair formation by the two inhibitors is the inactivation, or unreplaced loss, of pili.

A first test of the consistency of this interpretation was to compare the MS-2 adsorbing abilities of donor cells pretreated for two hours with CAP with that of untreated controls. CAP was added to log-phase cultures of MSC 603 in Pennassay broth (PB) and 1 ml samples removed immediately, and after 120 minutes, to test their ability to adsorb phage. An 0.1 ml quantity of PB containing 3×10^5 MS-2 phage particles was added to each sample. These adsorption mixtures were then sampled just after adding phage and at two minute intervals for ten minutes, and the number of unadsorbed phage determined as described in Methods. Control tubes containing no cells were assayed for

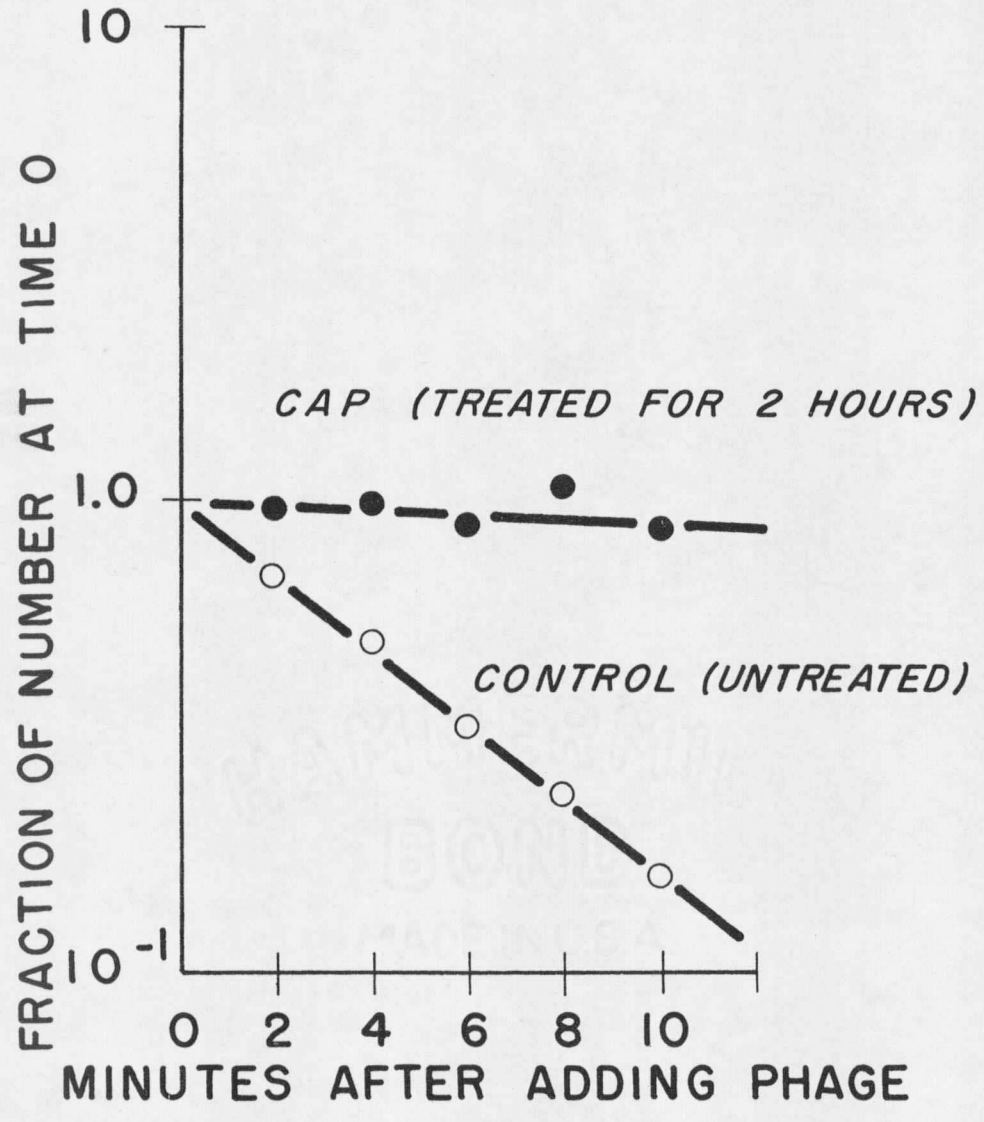

Figure 7. The effect of chloramphenicol (CAP) upon MS-2 adsorbing ability. 10^5 phage/ml were added to a log phase PB culture of MSC 603, and to a similar culture pretreated for 2 hours with CAP, at times 0. The number of free phage remaining was assayed at 2 minute intervals as described in Methods. All points are averages of three independent experiments.

plaque-forming units at 0 and 10 minutes. From the results (Figure 7) is appears that a two hour CAP pretreatment drastically reduces the number of phage adsorbing pili.

A second group of experiments was performed to compare the kinetics of decay of phage-adsorbing ability with the decay of fertility, in both CAP and PEA. Experiments measuring phage-adsorbing ability were performed as follows: CAP and PEA were added to aliquots of a log-phase culture of MSC 603 in PB. One ml samples were removed immediately (0 time) and added to tubes containing 0.1 ml of PB containing 3×10^5 MS-2 particles. After incubation for 15 minutes, the number of unadsorbed phage was assayed. This operation was repeated at 30 and 60 minutes after adding the inhibitors. Experiments measuring fertility were performed as follows: After adding PEA and CAP to log-phase PB cultures of MSC 603, samples were removed at 0, 30 and 60 minutes and mated with W1177 cultures also grown in PB. The criterion of fertility in these experiments was the $\underline{lac}^+$ recombinant yield after 30 minutes of mating. This is equivalent to the 60 minute yield (i.e., it is terminal) since the withdrawal effect does not occur under these cultural conditions, as observed first by deHaan and Gross (1962). Thus, the decay of pair forming ability measured here represents the decay of stable pair formation only.

These experiments (Figure 8) show that PEA (as well as CAP) pretreatment inhibits stable pair formation, a point not clearly established before. Although both inhibitors effect a decay in both

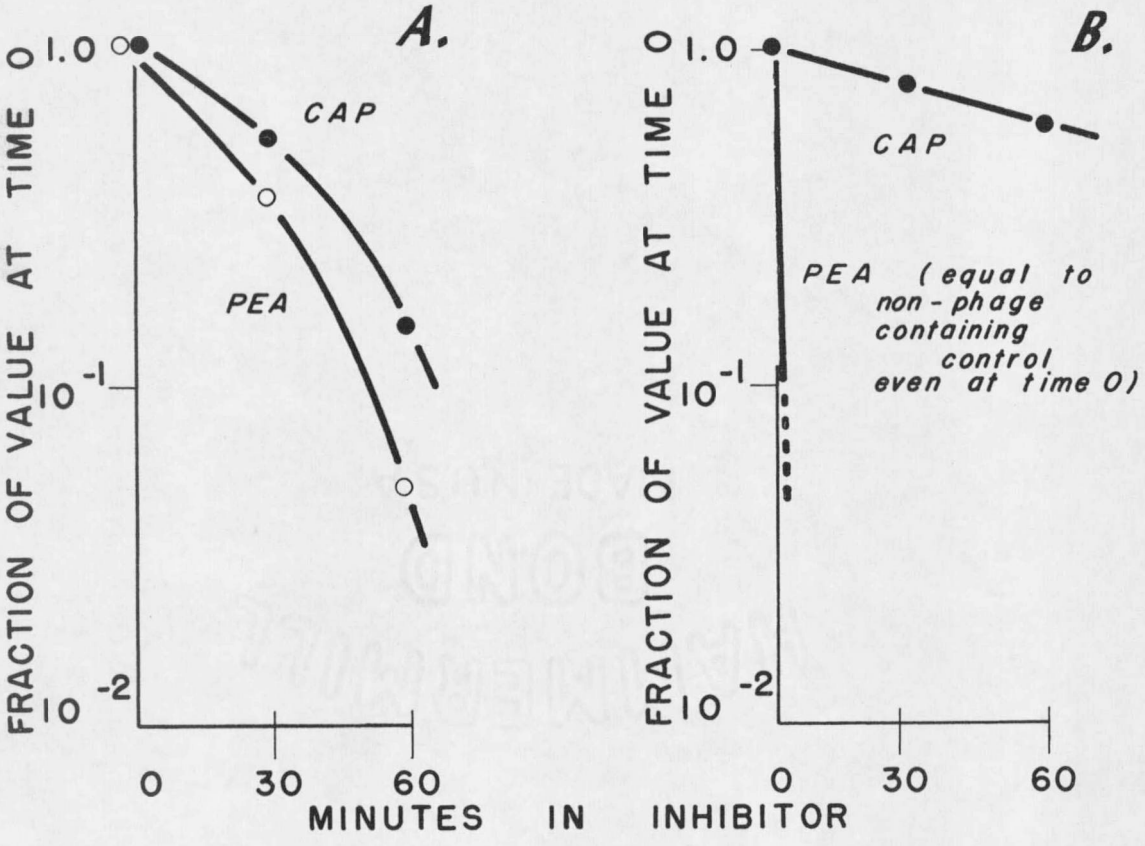

Figure 8. The decay of pair forming and MS-2 adsorbing abilities in the presence of inhibitors. Chloramphenicol (CAP) and phenethyl alcohol (PEA) were added to log phase cultures of MSC 603 in PB and samples removed at 0, 30, and 60 minutes to measure pair-forming and phage adsorbing abilities as described in the text. All points are averages of three independent experiments. A. Decay of pair-forming ability; B. Decay of phage-adsorbing ability.

properties studied, the four decay rates may all be unique. PEA causes a more rapid decay of both properties, but the difference between the two inhibitors' effects upon phage adsorption is far more apparent than the difference in their effects upon pair formation. The extreme speed of the PEA effect on phage adsorption is like that of its effect upon unstable pair formation in TG* medium, suggesting that F-pili are involved in the formation of unstable pairs. This rapid effect also strengthens the interpretation that PEA acts upon the cell surface, but does not necessarily mean that F-pili are the site of action.

A plausible interpretation of the difference between the effects of each inhibitor upon the two properties (e.g., the different rates of decay of pair formation and phage adsorbability after treatment with PEA) is that F-pili contain at least two components. The component which is involved (mainly) in stable pair formation would be sensitive to the action of PEA and to CAP by some common mechanism, most likely protein synthesis. The component involved (mainly) in phage adsorption (and perhaps unstable pair formation) would be relatively insensitive to inhibition of protein synthesis, but extremely sensitive to the rapid change in the cell surface produced by PEA. Alternatively, one might invoke two kinds of F-pili, or another surface structure, to explain these differential effects.

The effects of MS-2 phage adsorption upon gene transfer

The results of the preceding section are consistent with the

hypothesis that the F-pili are involved in the formation of conjugal pairs. They do not, however, provide any evidence about subsequent pilus function. Thus, the suggestion of Brinton et al. (1964) that F-pili might play a direct role in chromosome transfer remains unsupported.

In an attempt to test for the direct involvement of F-pili in transfer, experiments were performed to study the effect of adding MS-2 phage to mating mixtures in a situation where further pair formation is minimized. Any effect upon recombinant yield observed would be by interference with some pilus function occurring after pair formation. A complication in such an experiment is that infecting phage might kill the donor cells, or possibly the F^- cells (Sironi et al., 1964). The experiments, therefore, were performed at a temperature where MS-2 does not kill normally sensitive cells. A parallel control experiment was run at a temperature where the fraction of the population killed should be high.

The two temperatures were chosen on the basis of experiments in which aliquots of a suspension of MSC 603 were added to a number of nutrient agar plates, half of which had just been seeded with about 10^8 MS-2, a concentration far beyond that required to give confluent lysis in routine assays. Pairs of plates, one with and one without phage, were incubated overnight at different temperatures and the resulting colonies counted. If the plates were warmed (to the temperature at which they would later be incubated) before plating, differences

in the numbers of colonies appearing at the different temperatures were noted. At 34° C, there was no indication of killing; while at 40° C about 75% of the cells were killed.

Therefore, MSC 603 and W1177 were mated in PB at 37° C. After allowing five minutes for pair formation, the mating culture was diluted into four tubes: One containing a 10^{-2} dilution of PB in saline which had been prewarmed and maintained at 34° C, a second like the first but also containing 3×10^9 MS-2 particles/ml, a third like the first but prewarmed and maintained at 40° C, and a fourth like the second but prewarmed and maintained at 40° C. The kinetics of $\underline{lac}^+$ transfer were followed as in the TG* matings described earlier. Viable cells were assayed by diluting and plating on EMB-lactose from samples taken at 5 and 50 minutes.

Unexpectedly, no killing could be detected after 50 minutes at either temperature. Presumably, this was due to the suspending medium. Despite the absence of killing, the same sort of inhibition of transfer was observed at both temperatures (Figure 9). Hence it appears that MS-2 adsorption to already mated cells does inhibit recombination, and that this inhibition is not by way of killing. That this result might be produced by an effect of MS-2 upon host cell syntheses necessary for transfer, but not connected with F-pili, is unlikely. In experiments using a similar phage, f_2, it was found that even under conditions where the cells are killed no marked effects on host cell metabolism are seen until 15 to 20 minutes after infection (Lodish,

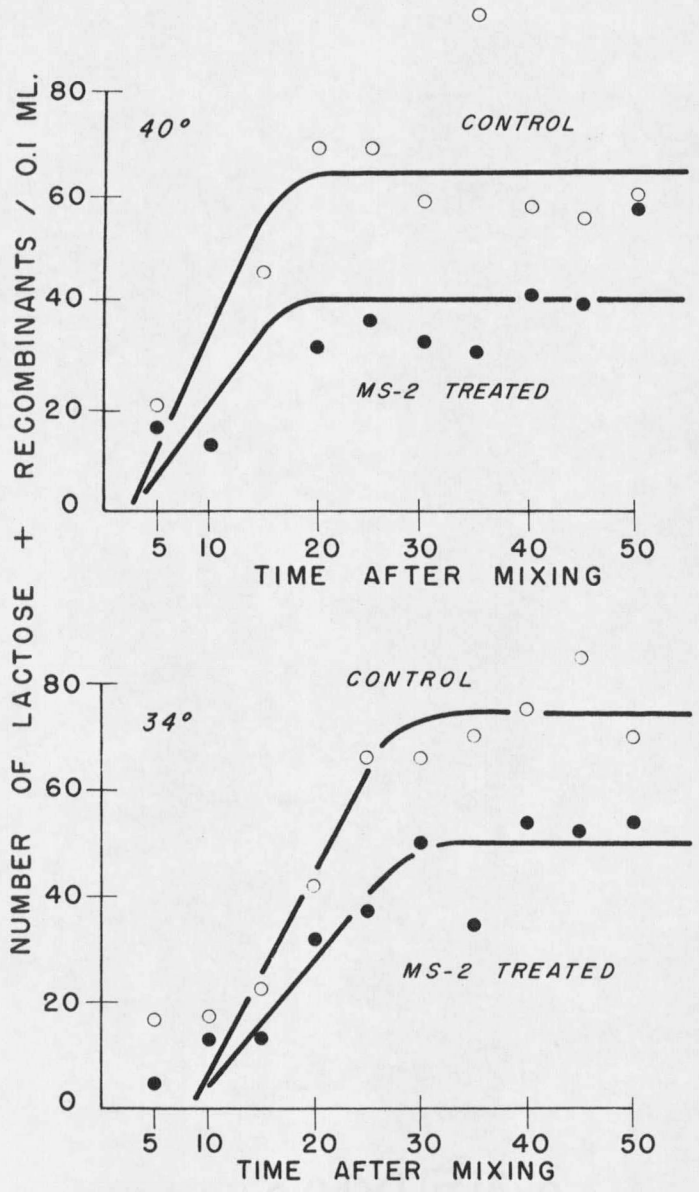

Figure 9. Inhibition of transfer by phage MS-2. Log phase cultures of MSC 603 and W1177 were mated at 37° and diluted into 10^{-2} PB with and without phage at 40° and 34° as described in the text. Mating cultures were sampled at five minute intervals to follow the transfer of the <u>lac</u>$^{+}$ marker. All points are single determinations.

et al., 1964), an interval corresponding to 20-25 minutes in the experiment described above. By this time the transfer of Lac^+ is almost complete. Instead, it appears that an event early after MS-2 adsorption is involved. Since such early events would be primarily pilus-associated, this result supports the hypothesis that F-pili are the corridors or vehicles of chromosome transfer.

DISCUSSION

The role of DNA synthesis. The results of the thymine deprivation experiments described here imply that, if concurrent DNA synthesis is required for chromosome transfer in *E. coli*, transfer-associated DNA synthesis must be slower than the DNA synthesis in an unmated cell. This agrees with the finding that the minimum time required for the transfer of a complete genome is one and one-half times longer than the cell generation time in the same medium (Taylor and Thoman, 1964). However, whether the rate of DNA synthesis in a thymine deprived Hfr cell is sufficient to transfer the chromosome at this speed remains an open question.

Even if the rate of DNA synthesis in a thymine deprived Hfr were found to be sufficient for a slow rate of transfer, this would not imply a necessary connection between transfer and concurrent DNA synthesis. It might be, for example, that synthesis of the chromosome to be transferred is concurrent with transfer, but that there is a considerable lag between the synthesis of a marker and its transfer, such that a "piling up" of the newly synthesized chromosome occurs; the actual transfer of this chromosome, then, might be carried out by some other mechanism. If this were so, one might expect the inhibition of DNA synthesis to affect markers which are transferred "late" more dramatically than those transferred early, and that the later a marker is transferred the greater the effect would be. Such a result has, in fact, been observed in experiments testing the effect of adding

phenethyl alcohol (PEA) during transfer (Jacob et al., 1963). However, the evidence regarding the way in which PEA inhibits DNA synthesis is not consistent with this interpretation. The experiments of Treick and Konetzka (1964) suggest that PEA prevents the initiation of new cycles of DNA replication, while permitting the completion of those cycles already initiated. If so, PEA should not affect those replication cycles which were initiated before its addition, and, in the experiments of Jacob et al., would not be expected to affect transfer. The finding that transfer is affected, therefore, suggests that transfer may be driven by some mechanism separate from DNA synthesis.

Consistent with these arguments are the experiments of Suit et al. (1965) which also suggest that transfer may be independent of DNA synthesis. DNA synthesis was brought to a complete halt in a culture of a histidine-requiring Hfr strain by two hours of histidine deprivation. Even so, when a sample of the culture was mated, normal transfer occurred. It was observed, however, that the final number of recombinants formed by the histidine deprived cells was greater than that formed by the same number of cells in a control mating. This seems inconsistent with our finding that treatment with protein synthesis inhibitors decreases the ability of Hfr cells to form pairs, but part of the difference may be accounted for by a difference in mating techniques. In the experiments of Suit et al., matings were performed by bringing Hfr and F^- cells into contact on the surface of membrane filter pads at densities which, therefore, far exceed those

used in our experiments. It may be that, at such close quarters, even damaged F-pili, or F-pili stubs, may suffice for pair formation; or it may be that F-pilus mediated pair formation is unnecessary at such high cell densities. This difference in technique, however, cannot explain the increase in the final number of recombinants observed in the experimental culture. But, we have predicted precisely this kind of effect of protein synthesis inhibition on the basis of Model 1 (cf. Results). Thus, a plausible interpretation of the increase is that, even under conditions where a high probability of contact is imposed, not all Hfr cells will engage in transfer unless they have been brought to the stage in replication at which transfer is most quickly accomplished.

As we have seen, the results of Suit et al. (in accord with the thymine deprivation results given here) indicate that transfer may occur independently of DNA synthesis, and further suggest that transfer is initiated between cycles of replication. Roeser and Konetzka (1964) believe that their experiments with PEA also connect the initiation of transfer with events occurring between replication cycles, specifically with the initiation of replication. This interpretation is questionable, however, since normal transfer was observed in matings made with Hfr cells removed from PEA in the middle of what they refer to as a "controlled (DNA) replication cycle". On their own interpretation of the action of PEA, none of these Hfr cells would have yet completed one such controlled cycle; hence, none should be able to

initiate another. Thus, their PEA experiments, like those of Jacob et al. described previously, suggest that the inhibition of transfer by PEA is via some mechanism other than DNA replication.

The role of the F-pili. The experimental evidence examined in the preceding section is all consistent with three conclusions: (1) That chromosome transfer is independent of DNA synthesis, but (2) that transfer is related to DNA synthesis in the sense that it can only be initiated at a particular time in the replication cycle, and (3) that PEA may affect processes more directly involved in transfer than DNA replication.

It is our contention that processes involved in F-pilus metabolism and necessary for transfer, may be those affected by PEA. An experiment implicating F-pili as agents of transfer, in addition to pairing has already been discussed. Likewise, it has been shown that PEA (and CAP) can affect two other functions of F-pili, namely pair formation and male-specific phage adsorption. Many questions raised by these experiments remain unanswered. For example, it is not clear how the inhibitors produce these effects, nor what the difference in effects between the two inhibitors may represent. Nonetheless, the idea that an F-pilus associated metabolic process is involved in chromosome transfer is supported by the finding (Wendt, unpublished) that the decay of pair-forming ability provoked by phenethyl alcohol occurs at the same rate in both TG* and PB media. It is a curious fact that the rate of gene transfer is the same in minimal medium and

broth, whereas the generation time is far longer in minimal medium (Compare, for example Jacob and Wollman, 1961, with Jacob et al., 1963). This indirect discovery of an F-pilus associated process which also occurs at the same rate in both kinds of medium suggests that an examination of the biochemistry of F-pilus synthesis might reveal both a peculiar metabolism, and the process which drives chromosome transfer.

SUMMARY

The consequences of two different (but not mutually exclusive) models of chromosome transfer by Hfr donors of E. coli have been examined experimentally. The finding that normal gene transfer can be carried out by Hfr's undergoing thymineless death in the presence of submaximal concentrations of thymine does not support the proposal that transfer depends on concurrent DNA synthesis. An attempt to stimulate the fertility of Hfr cultures by bringing all the Hfr to the (hypothetical) point at which transfer may be initiated by the use of chloramphenicol was unsuccessful. However, chloramphenicol treatment was found to reduce the ability of Hfr cells to form both stable and unstable pairs. These effects, and similar effects of phenethyl alcohol, have been traced to their action on donor-specific surface structures known as F-pili. The inhibition of gene transfer by the male-specific bacteriophage MS-2, which is believed to attack the donor cell via its F-pili, supports the idea that these pili are the corridors, or vehicles, of transfer. Finally, an examination of the available experimental evidence suggests that transfer may, in fact, be driven by processes associated with F-pili metabolism.

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